

# Comparative analysis of gene expression profiles of papillary thyroid microcarcinoma and papillary thyroid carcinoma

## ABSTRACT

**Purpose:** Papillary thyroid carcinomas (PTCs) measuring 1.0 cm or less were separately defined as papillary thyroid microcarcinomas (PTMs) by the World Health Organization, emphasizing on their benign behavior. However, some reported that PTMs may have aggressive behavior, can cause regional, or even distant metastases. But till now, the characteristics of PTMs were only reviewed and described by the clinicopathological parameters, and no analysis of PTM by the gene level is available. We report on the gene expression profiles of PTMs by the oligonucleotide microarrays and the results of comparative analysis with those of PTCs.

**Materials and Methods:** The gene expression profiles of 25 pairs of PTMs and their normal thyroid tissue counterparts, and 11 pairs of PTCs and their normal counterparts, were analyzed by Affymetrix Human Genome U133A. Data were analyzed by the SAM and the DAVID 2008 program to detect differentially expressed genes in supervised sample classification.

**Results:** Two-hundred thirteen statistically significant up-regulated genes and -183 significant down-regulated genes of PTMs compared with their normal counterpart thyroid tissues, which were mainly cell adhesion-related genes and immune response genes, were detected. Two-hundred sixty-one up-regulated and -157 down-regulated genes of PTCs were also detected. In the comparative analyses of gene expression profiles of PTMs and PTCs, no significant difference was found.

**Conclusion:** PTM should not be considered as the simple occult indolent thyroid cancer, but as the earlier stage of disease which eventually evolves into PTC, because the gene expression profiles of PTMs were not different from those of PTCs.

**KEY WORDS:** Papillary thyroid microcarcinoma, papillary thyroid carcinoma, gene, microarray

## INTRODUCTION

Thyroid carcinoma represents 1% of all malignant diseases and accounts for nearly 90% of neuroendocrine malignancies. Papillary thyroid carcinomas (PTCs) are the most common malignant thyroid tumors, representing 80-90% of thyroid malignancies. According to the World Health Organization (WHO), PTCs measuring 1.0 cm or less in diameter are separately defined as papillary thyroid microcarcinomas (PTMs), emphasizing on their benign behavior, probably of little clinical significance, which do not affect patients' survival.<sup>[1,2]</sup>

The clinical behavior of PTMs is usually indolent as it was considered historically. In one series,<sup>[3]</sup> 93% of the patients were free of disease during a follow-up of 3–23 years, with a mean of 6.3 years, and there was no single instance of distant metastases. However, rarely PTMs may have aggressive behavior,<sup>[4]</sup> can cause locoregional recurrences,<sup>[5,6]</sup> and cervical lymph node metastases.<sup>[7-9]</sup> The lack of long-term randomized prospective studies makes it very difficult to establish which therapeutic

approach is better and explains the present uncertainty and controversies for the management of PTMs.

The genome-wide microarray analysis enabled us to obtain comprehensive gene expression profiles related to phenotypic and biological information in cancer cells<sup>[10-16]</sup> and to identify multiple applicable targets for the development of novel anti-cancer drugs and/or diagnostic markers. For the thyroid carcinoma, only a few reports are available on the results of this novel approach, mainly focusing on the identification of unknown molecules involved in the carcinogenic pathway especially from the differentiated carcinoma to the anaplastic carcinoma, and the identification of the new markers to discriminate the follicular carcinoma and the benign follicular adenoma.<sup>[11,17-20]</sup>

All the genome-wide microarray analyses of PTCs to date is those of the relatively large size tumors, that is larger than 2 cm in diameter, and there is no result available on the gene expression profiles of PTMs which might show different clinical behaviors

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from PTCs. Thus we performed the oligonucleotide microarray analysis of the PTMs to reveal the gene expression profiles of PTMs and compared the results with those of the PTCs to give valuable information on the decision of the therapeutic approaches to the PTMs.

## MATERIALS AND METHODS

This study was approved by the institutional review board of the Seoul National University Hospital, Seoul, Korea (registered number: H-0706-048-211).

Eighty PTC and PTM tissue samples, along with 80 normal thyroid tissue samples were obtained with written informed consent from the patients undergone total thyroidectomy and neck dissection under the diagnosis of papillary carcinoma at the Department of Surgery, Seoul National University Hospital. These cancer tissues were histopathologically diagnosed as classic papillary carcinoma, and atypical variants such as tall cell, columnar cell, insular, or follicular variants were excluded.

The 0.2 cm sized cube of cancer tissue was obtained from the center of the cancer lesion immediately after the lobectomy of the pathologic lobe of the thyroid gland, and the 0.2 cm sized cube of normal thyroid tissue was obtained from the center of the normal contralateral lobe in the same patient just after the completion of the contralateral lobectomy. Each samples were embedded in RNA later solution immediately after the sampling and stored at 4°C overnight for the isolation of RNA in the next day.

After the isolation of RNA, the purity and quality of the samples were evaluated with a ND-1000 spectrometer (Nanodrop Technologies) and Bioanalyzer Nano Labchips (Agilent Technologies). When the RNA signal pattern was identical to that of the control sample and the ratio of 28s/18s rRNA was more than 1.0, the paired cancer and normal thyroid tissues were included and undergone the microarray procedure, otherwise excluded. Finally 72 paired cancer and normal tissues from the 36 patients, including 50 PTM along with normal tissues from the 25 PTM patients and 22 PTC along with normal tissues from the 11 PTC patients, were included for the study.

The Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays were used for microarray hybridizations. This GeneChip comprises >54,000 probe sets and analyzes the expression level of >47,000 transcripts. For microarray hybridization, we followed the protocol described in the Affymetrix GeneChip eukaryotic two cycles target preparation protocol (Affymetrix). For the first-round synthesis of double-stranded cDNA, 100 ng of total RNA were reverse transcribed using the two-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA) and T7-oligo-dT primer according to the manufacturer's instructions followed by IVT amplification with the MEGAscript T7 kit (Ambion, Inc., Austin, TX). After cleanup of the cRNA with a

GeneChip sample cleanup module IVT column (Affymetrix), second-round double-stranded cDNA was amplified using the IVT labeling kit (Affymetrix). A 20 µg aliquot of the labeled product was fragmented by heat and ion-mediated hydrolysis at 94°C for 35 min in H<sub>2</sub>O and 8 µl of 5× fragmentation buffer (Affymetrix). The fragmented cRNA was hybridized for 16 h at 45°C in a hybridization oven 640 to a U133 Plus 2.0 oligonucleotide array (Affymetrix). The washing and staining of the arrays with phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR) were completed in a fluidics station 450 (Affymetrix). The arrays were then scanned using a confocal laser GeneChip scanner 3000 (Affymetrix). For the data analysis, global normalization at a target value of 500 was applied to each of the 72 arrays under consideration using the GeneChip operating software (Affymetrix). Normalized data from text files were imported to a Microsoft Excel spreadsheet.

To find out the commonly over- or under-expressed, in other words, up- or down-regulated genes in the cancer tissue compared with normal tissue through the intra-individual analyses, we performed significance analysis of microarrays (SAM, Version 3.0, 2007, [www-stat.stanford.edu/~tibs/SAM/](http://www-stat.stanford.edu/~tibs/SAM/)). After the identification of the statistically significant genes from the SAM plotsheet and Delta table, we performed functional annotation bioinformatic analysis using the Database for Annotation, Visualization and Integrated Discovery 2008 (DAVID 2008, [david.abcc.ncifcrf.gov](http://david.abcc.ncifcrf.gov)) program, and selected the statistically significant genes by their function and names with a Benjamini cutoff value <0.05. Then the nonsupervised hierarchical clustering was performed.

## RESULTS

The gene expression profiles of the 72 paired cancer and normal tissues from the 36 patients, including 50 PTM along with normal tissues from the 25 PTM patients and 22 PTC along with normal tissues from the 11 PTC patients, were analyzed. The mean patient age was 51.3 years (range, 33-71). There were 25 females and 11 males. The mean tumor size was 0.92 cm (range, 0.3-2.4). Regional lymph node metastasis was observed in 16 patients, extrathyroidal extension of the cancer in 24 patients, and multifocal cancer in 12 patients. Overall, 12 patients were diagnosed as stage I and the other 24 patients were stage III, according to the American Joint Committee on Cancer Staging, 6th edition [Table 1].

Among the 25 PTM patients, regional lymph node metastasis was observed in 10 patients, and TNM stage I was in 10 patients, and III was in 15 patients. Multifocal cancer was observed in 8 patients out of 25 PTM patients. Among the 11 PTC patients, regional lymph node metastasis was observed in 6 patients, and TNM stage I was in 2 patients and III was in 9 patients. Multifocal cancer was observed in 4 patients out of 11 PTC patients.

In the analysis of the gene expression profiles of PTMs, after the

**Table 1: Clinicopathologic features of the cancer samples used for microarray analyses**

Characteristics	Value
Patient's age (yrs)	51.3±10.7 (range, 33-71)
Patient's gender (n, male: female)	11: 25
Tumor size (cm)	0.92±0.43 (range, 0.3-2.4)
Diagnosis (n)	
Papillary thyroid microcarcinoma	25 (69.4%)
Papillary thyroid carcinoma	11 (30.6%)
Lymph node metastasis (n)	16 (44.4%)
Extrathyroidal extension (n)	24 (66.7%)
Multifocality (n)	12 (33.3%)
TNM stage* (n)	
Stage I	12 (33.3%)
Stage III	24 (66.7%)

According to the American Joint Committee on Cancer Staging, 6<sup>th</sup> edition.

SAM, statistically significant 1688 up-regulated genes and 2543 down-regulated genes, in total 4231 genes, were obtained at the Delta value 3.05 and false discovery rate (FDR) < 0.05 from the PTMs and their paired normal thyroid tissue array results.

After the functional annotation analysis of these genes using the DAVID program, 213 significant up-regulated genes in PTMs, which belongs to the 12 functional categories described in [Table 2], were selected at the Benjamini cutoff value <0.05. In turn, 183 significant down-regulated genes in PTMs, which belongs to the four functional categories described in Table 3, were also selected at the Benjamini cutoff value <0.05. The lists of significant genes selected are not shown here owing to the limited space of the journal.

In the analysis of the gene expression profiles of PTCs, as in the analysis of PTM profiles, after the SAM, statistically significant 1319 up-regulated genes and 1742 down-regulated genes, in

**Table 2: Functional categories of genes up-regulated in papillary thyroid microcarcinoma**

Cluster	Count	P-value	Benjamini
Collagen	23	2.5E-5	1.1E-3
Cytoskeletal protein binding	61	1.2E-5	3.9E-3
Integrin	14	2.3E-5	1.0E-3
GTPase regulator activity	56	1.9E-5	6.1E-3
Tyrosine-protein kinase	23	4.4E-5	1.7E-3
Transmembrane receptor protein tyrosine kinase activity	17	1.4E-4	3.3E-2
Trimer	13	4.0E-7	3.0E-5
Triple helix	13	3.2E-6	2.3E-4
Hydroxylysine	13	4.6E-6	2.7E-4
EF hand	13	8.2E-4	2.0E-2
Phosphoric monoester hydrolase	15	7.8E-4	1.9E-2

**Table 3: Functional categories of genes down-regulated in papillary thyroid microcarcinoma**

Cluster	Count	P-value	Benjamini
FAD binding	20	2.9E-5	1.4E-3
Flavoprotein	30	1.8E-6	1.5E-4
Methyltransferase activity	33	3.5E-4	4.4E-2
Oxidoreductase	98	8.3E-7	7.4E-5

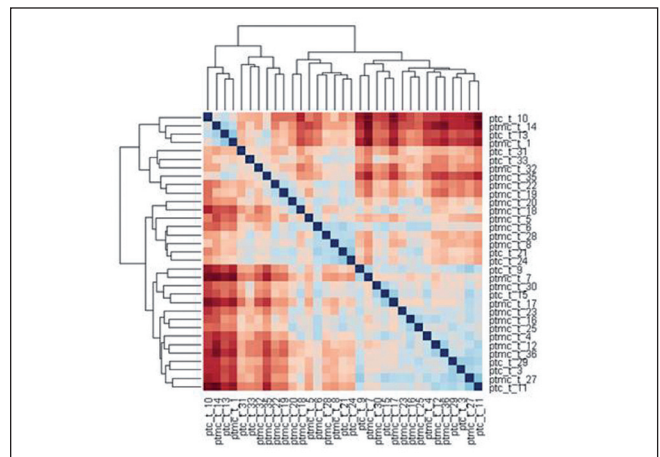
total 3061 genes, were obtained at the Delta value 1.70 and FDR < 0.05 from the PTCs and their paired normal thyroid tissue array results.

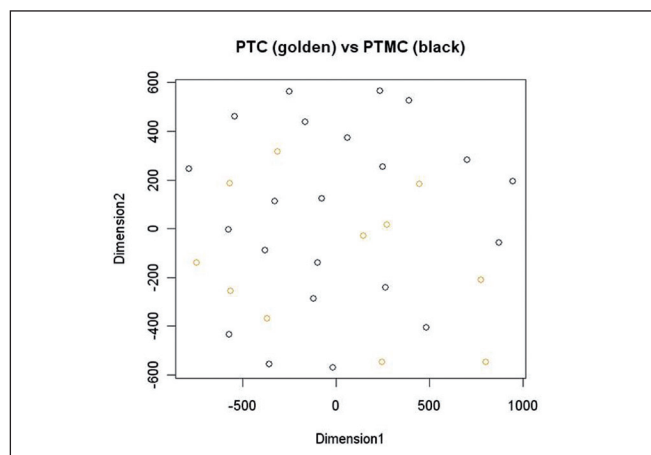
And after the functional annotation analysis of these genes using the DAVID program, 261 significant up-regulated genes in PTCs, which belongs to the 13 functional categories, were selected at the Benjamini cutoff value <0.05. In turn, 157 significant down-regulated genes in PTCs, which belongs to the 7 functional categories, were also selected at the Benjamini cutoff value <0.05. The lists of significant genes and functional categories selected are also not shown here.

To evaluate the difference between the gene expression profiles of PTMs and PTCs, we applied several analytic methods in the following.

First, Figure 1 is the heatmap of the dissimilarity matrix between PTMs and PTCs. We selected 1825 gene probe sets from the microarray data in which variations of the expression signal intensities between the samples were maximal, and colorized the distance between the samples using those genes. If the similarity of gene expression profiles between the samples increases, the distance between the samples is small, and it is colored blue in the heatmap. If the similarity between the samples decreases, it turns red. As seen in the figure, the similarity of the samples does not correlate with the classification of PTM and PTC. In other words, PTMs and PTCs could not be differentiated by gene expression profiles.

Second, Figure 2 is the two-dimensional projection of distances between PTMs and PTCs. We projected the results of the distance matrix of the samples in two dimensions, and if the similarity of gene expression profiles between the samples increases, the samples shall be located closer in the projection. PTMs are indicated with black circles and PTCs with golden circles in the figure. PTMs and PTCs do not make any cluster and located irregularly after the projection as in

**Figure 1: Heatmap of the dissimilarity matrix between papillary thyroid carcinoma and papillary thyroid microcarcinoma samples**



**Figure 2:** Two-dimensional projection of distances between papillary thyroid carcinoma and papillary thyroid microcarcinomas

the figure, assuming that the similarity of the samples in the gene expression level does not correlate to the PTM/PTC classification.

Third, Figure 3 shows the four different dendrograms of the samples from the various hierarchical clustering algorithms – complete linkage, single linkage, Ward method and divisive clustering. Cluster in the dendrogram represents the relative similarity of the gene expression profiles of the samples included in. As shown in the figure, even though using the various different methods, PTMs and PTCs could not be divided by the gene expression profiles.

In Figure 4, statistical significance of each cluster from the Ward method hierarchical clustering described in Figure 3 is expressed on multiscale bootstrap resampling. The numbers indicate two statistical supporting values: red number denotes AU (Approximately Unbiased) value and green number indicates BP (Bootstrap Probability) value. The clusters with AU larger than 95% are highlighted by rectangles, which

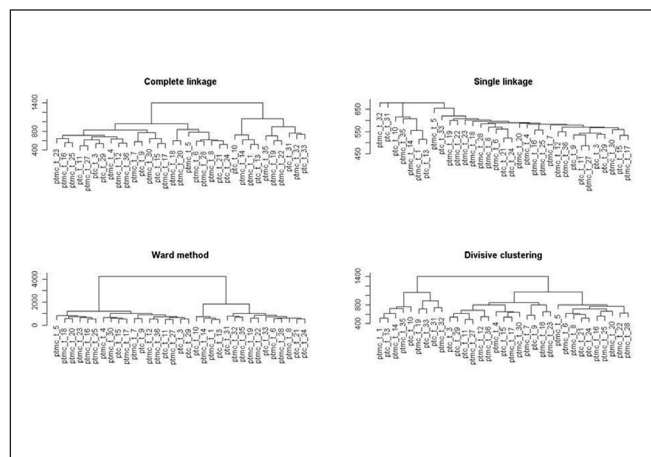
are strongly supported by data. In the rectangle significant clusters, PTMs and PTCs coexist, also implying that PTMs and PTCs cannot be divided by the gene expression profiles.

## DISCUSSION

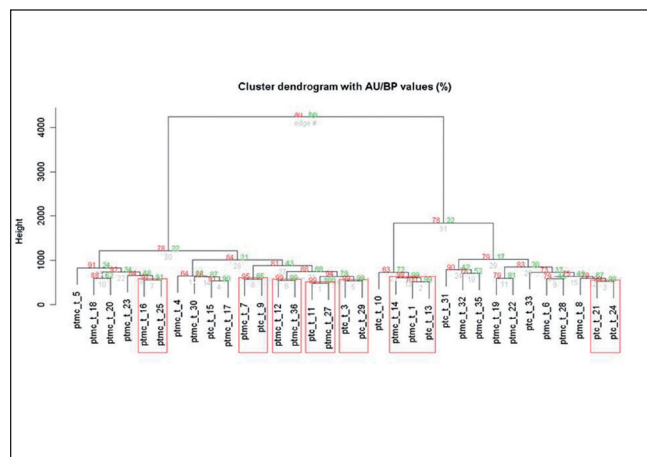
Recently, the proportion of PTMs increased rapidly owing to widespread use of thyroid ultrasonography. It has been considered that PTM shows a benign behavior and has a high likelihood of remaining silent during the lifetime. However, several recent studies showed that the recurrence rate of PTM was not negligible and there existed cases with distant metastases or fatal outcome.

Noguchi, *et al.*, from an analysis of 867 patients affected by PTM, concluded that total thyroidectomy is not necessary and that modified radical lymph node dissection is not necessary unless macroscopic lymph node metastases are present.<sup>[21]</sup> Ito, *et al.* addressed the issue of whether operation for PTMs is needed.<sup>[22]</sup> They started an observational trial in 162 patients with PTM diagnosed by FNAC who volunteered not to have surgery and found that more than 70% of tumors either did not change or decreased in size with respect to baseline. In 10% of the cases the tumor diameter increased above 10mm, and in 1.2% of the cases lymph node metastases appeared during follow-up. In the control group of 626 patients with PTM who were treated by surgery, 50.5% had lymph node metastases and multifocality was present in 42.8%; during the follow-up, the recurrence rate was of 2.7% at 5 years and 5.0% at 8 years. These authors concluded that PTM was frequently not progressing and that patients could choose the observation.

In contrast with these Japanese authors, Hay, *et al.* performed a multivariate analysis of 535 PTM patients with a mean follow-up of 17.5 years and reported that 27 patients had recurrence, and the authors found that risk factors for locoregional recurrence were lymph node metastases at presentation and extent of initial thyroid surgery.<sup>[23]</sup> Baudin, *et al.*, in their

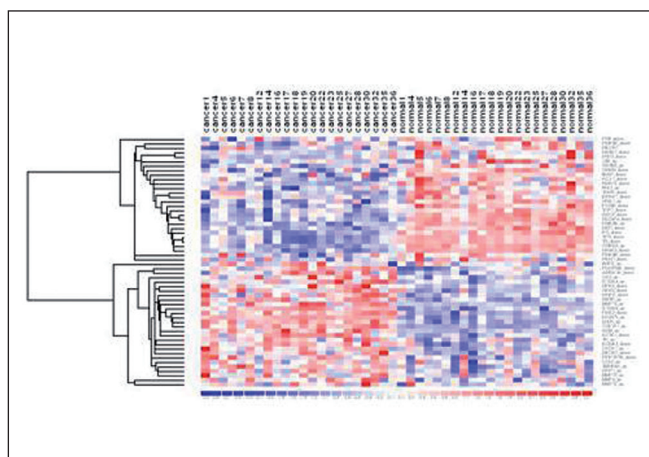


**Figure 3:** Four different dendrograms, clustering of papillary thyroid carcinoma and papillary thyroid microcarcinoma



**Figure 4:** Hierarchical clustering based on multiscale bootstrap resampling





**Figure 5:** Hierarchical clustering of papillary thyroid microcarcinomas and their counterpart normal thyroid samples using previously reported papillary thyroid carcinoma-specific 67 genes

multivariate analysis, confirmed that multifocality and extent of initial surgery were significant risk factors for recurrence.<sup>[9]</sup> Both these studies therefore underlie the importance of total or near-total thyroidectomy and lymph node dissection in PTMs presenting with lymph node metastases.

As above, many controversies on the management of PTMs coexist to date and it is very difficult to decide which therapeutic approach is better. This might not only be due to the absence of long-term randomized prospective studies, but also might be due to the lack of the information on the PTMs compared with PTCs in the molecular level.

Thus we analyzed the gene expression profiles of PTMs, which have never been reported before, and compared those with PTCs to provide the basic evidence for the treatment of PTMs.

Most of the commonly up-regulated and down-regulated genes in PTMs were functionally associated cell adhesion and cell-mediated immunity. These functional annotation analytic results of PTMs showed almost no difference in comparison with those in PTCs in this study.

Ezlinger *et al* recently meta-analyzed most of the previously published microarray-based gene expression profiles of thyroid carcinomas, and reported 67 significantly up-regulated and down-regulated genes commonly expressed in PTCs.<sup>[24]</sup> We used these genes for hierarchical clustering of our PTMs and their normal tissue counterparts [Figure 5]. As seen in Figure 5, cancers and normal tissues were well separately clustered implying that the quality of our collected tissue, the procedures of our microarray experiment and the resulted data were quite reliable. However, in direct comparison of the above previously reported 67 genes of PTCs and 261 significant genes of PTMs from our study, only 6 up-regulated genes (MMP13, TMSB4X, S100A4, S100A6, IGF2R, COL1A1) and 1 down-regulated gene (TPO) were matched. This might be due to the usage of old

generation microarray chips, such as Affymetrix GeneChip Human Genome U95 which lack in the number of transcript probes, in the most of the previous reports. Another important reason of this discrepancy might be due to the fact, as Ezlinger, *et al.* mentioned in their article, in most of the previous studies authors performed inter-individual (unpaired) analysis defining the reference tissue as non-nodular healthy tissue or benign tumor tissue which might be interfered by the bias caused by the difference between the individuals and the reference tissues. Compared with these studies, our data and results would be more reliable because we performed intra-individual (paired) analysis using the newest generation gene chips.

As shown in [Figures 1–4], PTMs and PTCs show no difference in gene expression level. Thus we can assume that PTM should not be considered as the simple occult indolent thyroid cancer, but as the earlier stage of disease which eventually evolves into PTC.

Most PTMs will slowly be progressed into PTCs; however, some PTMs will show aggressive behavior causing regional or even distant metastases in their earlier presentation. To date, we still cannot predict these biological behaviors of PTMs. Thus it would be important to find out the genes that may help to predict which of the PTMs will behave aggressively in the future study.

## CONCLUSION

PTM should not be considered as the simple occult indolent thyroid cancer, but as the earlier stage of disease which eventually evolves into PTC, because the gene expression profiles of PTMs were not different from those of PTCs.

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